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We are developing systems for genetic analysis in archaebacteria. Transfection and transformation conditions for Halobacterium volcanii have been optimized. High efficiency transformation can be obtained with plasmids and linear chromosomal DNA. Mevinolin resistance has been developed as a marker for plasmid selection, and shuttle vectors, selectable and maintainable in both H. volcanii and Escherichia coli have been constructed and characterized.

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Annual Progress Report on Contract N00014-88-J-1030, R&T 2441d014

Principal Investigator:	W. Ford Doolittle
Contractor:	Dalhousie University Halifax, Nova Scotia
Contract Title:	Development of Shuttle Vectors for Halobacteria.
Start Date:	1 February 1988
Period of Performance:	1 February 1988 - 1 February 1989

Research Objective: To develop transformation systems for halophilic archaebacteria, construct a variety of useful halobacterial plasmid vectors including halobacteria - *E. coli* shuttle vectors, and construct suitable halobacterial host strains. We then wish to demonstrate the utility of these genetic tools with several model applications.

Progress (Year 1): The development of a useful system for molecular genetic analyses in halobacteria requires (*i*) easy, reproducible methods for the introduction of exogenous DNA (transformation), (*ii*) genetic markers permitting selection in one or more hosts, and (*iii*) plasmid vectors, including hybrid constructs which can replicate in both halobacteria and an alternate eubacterial or eukaryotic host ("shuttle vectors"). Progress has been made in all three areas, and we have started to use these methods to (*iv*) analyse gene structure and function in *Halobacterium volcanii*.

(i) *Transformation*

Transfection and general transformation methods. At the time of application, we had completed a study of transfection, using *Halobacterium halobium* and its phage ΦH. We showed (Cline and Doolittle, 1987) that, in the presence of polyethylene glycol (PEG), the 59 kbp double-stranded DNA genome of ΦH could be taken up and expressed by spheroplasts of *H. halobium* , giving plaques on lawns of sensitive cells when spheroplasts were allowed to regenerate in soft agar overlays. Efficiencies of transfection (10^7/μg DNA) and regeneration (up to 60-70%) are very high, and we also showed that ΦH DNA will transfect *H. volcanii* (which does not absorb phage particles), producing plaques titratable on *H. halobium* lawns. Uptake of DNA by both species appears to be about equally efficient, but DNA from *H. halobium*-grown phage is restricted in *H. volcanii*, which shows a 10^4 - 10^5 reduction in transfection efficiency (400 plaques per μg) but no reduction in burst sizes (Charlebois et al. 1987; Cline et al. 1989a,b).

Transfection procedures for both species have been optimized (Cline and Doolittle, 1987; Charlebois *et al.*, 1987; Cline *et al.*, 1989a,b) and we have developed a general protocol for transfection and transformation. It involves (i) resuspension of cells in spheroplasting solution (about half the NaCl compared to growth medium, no divalent cations, sucrose as an osmotic stabilizer, buffer, and a small amount of KCl), (ii) conversion of cells to spheroplasts by addition of EDTA, (iii) addition of transforming DNA, (iv) addition of PEG solution to induce uptake of DNA, (v) washing or dilution of spheroplasts with regeneration salts solution to reverse aggregation by PEG, and (iv) plating spheroplasts in regeneration overlay agar. Transformation protocols have been optimized for : MW and concentration of PEG (600 daltons and 30% for *H. halobium*, not re-optimized for *H. volcanii*); NaCl concentration (2 M for *H. halobium*, 0.8 M for *H. volcanii*);pH (8.75 for *H. halobium*, 8.2 for *H. volcanii*); length of incubation with DNA (5 min. for *H. halobium*, non-critical for *H. volcanii*); length of incubation with PEG (10 - 20 min. for *H. halobium*, non-critical for *H. volcanii*); phase of culture growth (late-log phase for *H.*

halobium, non-critical below stationary phase for *H. volcanii*), and number of cells per transformation (10^9 cells per 200 µl for *H. volcanii*) (Cline and Doolittle 1987; Cline *et al.*, 1989b). Phage ΦH transfections in either *H. halobium* or *H. volcanii* and transformations with genomic DNA in *H. volcanii* (see below) are linearly dependent on input DNA up to 1 µg.

Cells for transformation may be stored frozen at -70° C in spheroplasting solution plus 15% glycerol, at least for six months, and give transformation efficiencies comparable to fresh cells (Cline *et al.*, 1989a). This innovation has greatly facilitated experimental work.

Transformation with plasmid DNA. In work under way at the time of application, we showed that pHV2, the 6,354 bp endogenous *H. volcanii* plasmid we had sequenced, is taken up by and stably transforms the cured (pHV2-deficient) strain of *H. volcanii* (WFD11) that we had obtained after growth in ethidium bromide (Charlebois *et al.*, 1987). Initially, plasmid transformation could be demonstrated only by colony hybridization with labeled plasmid sequences as probe; efficiencies (10^7-10^8/µg DNA) were as high as for transfection. More recently, we have used genetic markers to select transformants (see below).

Transformation with linear chromosomal DNA fragments. Linear chromosomal DNA can also be used in transformation experiments, selecting for prototrophic transformants of auxotrophic mutants. We have perhaps twenty such mutants, mostly amino acid auxotrophs which we have found ourselves after replica plating of ethyl-methane-sulfonate-mutated *H. volcanii*, or which we have obtained from M. Mevarech. All can be transformed to prototrophy. Randomly sheared total wild-type genomic DNA of 35 kbp or larger transforms with efficiencies of up to 7×10^4 per µg, under the above conditions (Cline *et al.*, 1989b). As DNA size is reduced below 35 kbp transformation efficiency falls off. Based on estimates that 35 kbp represents about 1% of the *H. volcanii* genome, we calculate that transformation with a pure species of 35 kbp would give transformation efficiencies of around 10^7 per µg -- comparable to values obtained with plasmids and thus suggesting that recombination is efficient. Transformation with genomic DNA will prove useful for classical genetic techniques such as strain construction and fine mapping.

Transformation with cosmid DNA. In work supported by the Medical Research Council of Canada, we are preparing a "bottom-up" physical map of the *H. volcanii* genome, using restriction site information to link cosmid clones of *H. volcanii* DNA into "contigs". Clones representing at least 95% of the approximately 4,000 kbp genome have been linked so far, although there are gaps in the map. One of the more useful products of this effort is a minimally overlapping set of cosmid clones representing most of the genome, to which cloned genes can be mapped by hybridization. Relevant to the transformation work reported here is that cosmid DNA can, in spite of restriction, give rise to prototrophic transformants of auxotrophic strains. We have used this to localize genes for histidine and arginine biosynthesis to given cosmids in the collection, and then mapped those genes within cosmids by subcloning, or transformation with uncloned cosmid fragments taken directly from gels. Hundreds of prototrophic colonies can be obtained under conditions where there is little if any detectable reversion. The affected *his* gene in one mutant has been succesively localized to a small DNA fragment, which has been sequenced. The sequence shows strong homology to the *E. coli hisC* locus.

(ii) *Markers*

Mevinolin resistance. Few antibiotics effective against eubacteria are useful in archaebacterial genetics. Recently, Cabrera *et al.* (J. Biol. Chem. 261, 3578-3583, 1986) showed that the growth of *H. halobium* is exquisitely sensitive to mevinolin, an inhibitor of HMGCoA reductase, and that this enzyme is inhibited *in vitro* by the drug. *H. volcanii* is also sensitive, and we have used primarily mevinolin sensitivity/resistance as the basis for development of a selectable shuttle vector system (see section iii).

Other markers. A number of auxotrophs, requiring amino acids or purine or pyrimidine bases, are now available to us. We have shotgun cloned genomic DNA from wild-type *H. volcanii* into pHV2, and transformed auxotrophic mutants with ligation mixtures. We now have cloned wild-type loci corresponding to mutants in the tryptophan, leucine, asparagine, arginine, histidine, glutamate and proline pathways in this manner. At least in the case of tryptophan clones, plasmids are stable, and can be purified and used again in transformation. Sequence analysis has begun for one of these tryptophan clones

We have also identified a number of amino acid analogs, especially tryptophan analogs, which strongly inhibit the growth of *H. volcanii*. These could be used in the development of further selectable markers.

(iii) Construction of plasmid vectors (Lam and Doolittle, 1989)

Mevinolin resistant mutants arise at a frequency of about one in 10^9 colonies plated. MluI-digested DNA from such mutants was "shotgun" cloned by ligation into the single MluI site of a variant of pHV2, pHV51. (pHV51 contains a copy of the transposable element ISH51 [which includes the MluI site] inserted at about position 5,500, and was isolated fortuitously during random plasmid screening.) With an MluI shotgun library made in this way, mevinolin-resistant transformants of the plasmid-deficient strain WFD11 were obtained in good yield   Plasmids reisolated from them will transform *H. volcanii* to mevinolin resistance at frequencies in excess of $10^7/\mu g$. The structure of one of these plasmids, pWL2, is shown schematically in Fig. 1.

The pBR322 derivative pAT153 was cloned into pWL2 at several positions, in both orientations. Some of these constructs are shown in Fig. 1. Each can function as a shuttle vector, selectable in *E. coli* through ampicillin- or tetracycline-resistance, and in *H. volcanii* with mevinolin. Restriction endonuclease analysis and Southern hybridization experiments show that these constructs maintain the structure indicated in Fig. 1, through cycles of growth in the alternate hosts.

To make a vector more compact than the 19 kbp constructs in Fig. 2 (which would thus be less subject to restriction by *H. volcanii* and more easily taken up by *E. coli*), the further tailoring shown in Fig. 2 was undertaken. A 3.5 kbp SphI-KpnI fragment adequate to determine mevinolin resistance was derived in several steps from pE3+, and ligated into a derivative of pH455 from which the original 7.9 kbp mevinolin region had been removed, to give the 15 kbp pWL101. Various deletions were made into the pHV2 moiety of this construct, with the results shown in Fig. 3. So far the smallest shuttle vector that can be maintained and selected in both hosts is the 11.2 kbp pWL102. Insertions can be made into unique KpnI, XbaI, SphI, SpeI, ClaI and EcoRI sites without disrupting selection or maintenance. Even though pWL101 and pWL102 grown in *E. coli* suffer restriction in *H. volcanii*, a microgram of vector DNA prepared in *E. coli* will produce thousands of mevinolin-resistant *H. volcanii* transformants in a typical experiment, while on average less than one new spontaneously-resistant mutant appears on plates with comparable numbers (10^8) of regenerated spheroplasts after mock (no DNA) transformation.

Work plan (Year 2): As detailed (mostly) in our initial application, we will (i) select for *H. volcanii* mutants deficient in restriction endonuclease activity (which we can now easily do by selecting for high efficiency of double transformation), (ii) develop methods for insertional mutagenesis and recovery of eubacterial vectors integrated into the halobacterial genome (for which we now have evidence), (iii) determine, by sequencing, whether the mevinolin resistance determinant we are using is actually the HMGCoA reductase structural gene, (iv) develop, by *in vitro* synthesis, a supressor tRNA with which we can look for nonsense mutations among our collection, and (v) further show the utility of this approach by sequence characterization and mapping of some of the genes cloned by complementation of auxotrophic mutations.

Publications from this project (those marked with asterisk acknowledge ONR support -- preprints will be forwarded to ONR under separate cover)

Cline, S. W. and Doolittle. W.F. (1987) Efficient transfection of the archaebacterium *Halobacterium halobium. J. Bacteriol.* **169**, 1341-1344.

Charlebois, R.L., Lam, W.L., Cline, S.W., and Doolittle, W.F. (1987) Characterization of pHV2 from *Halobacterium volcanii* and it use in demonstrating transformation of an archaebacterium. *Proc. Natl. Acad. Sci. U.S.A.* **84**, 8530-8534.

*Cline, S.W., Lam, W.L., Charlebois, R.L., Schalkwyk, L.C. and Doolittle, W.F. (1988) Transformation methods for halophilic archaebacteria. *Can. J. Microbiol.* **35**, in press.

*Lam, W.L. and Doolittle, W.F. (1989) Shuttle vectors for the archaebacterium *Halobacterium volcanii.* Submitted to *Proc. Natl. Acad. Sci. U.S.A.*, 27 January.

*Cline, S.W., Schalkwyk, L.C. and Doolittle, W.F. (1989) Transformation of the archaebacterium *Halobacterium volcanii* with genomic DNA. To be submitted to *J. Bacteriol.* in February.

Inventions: none

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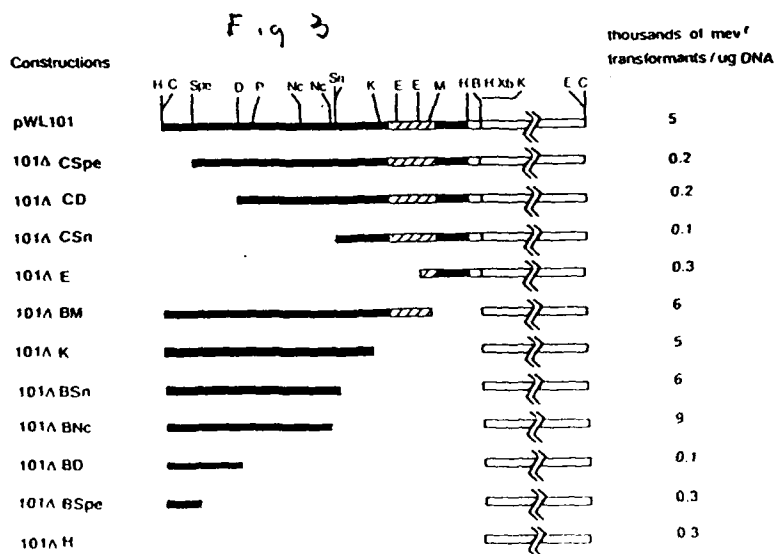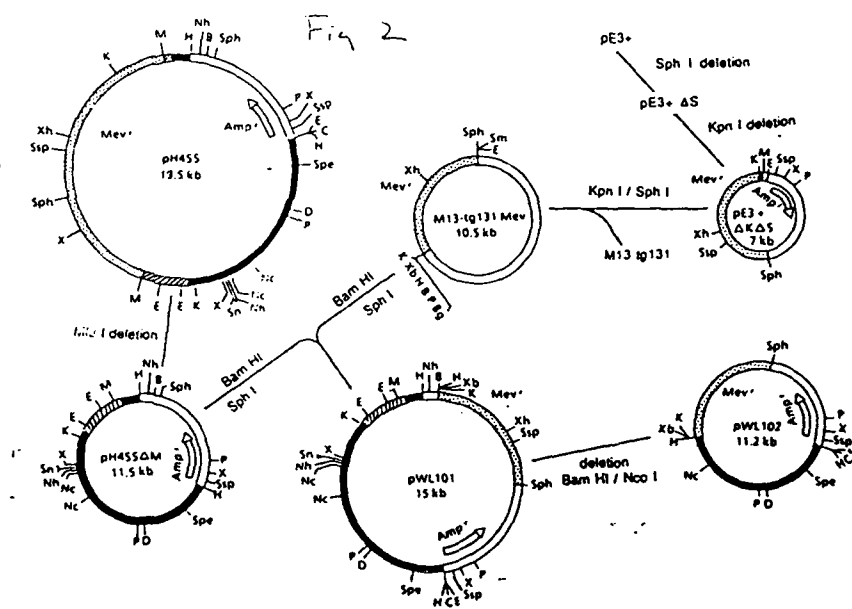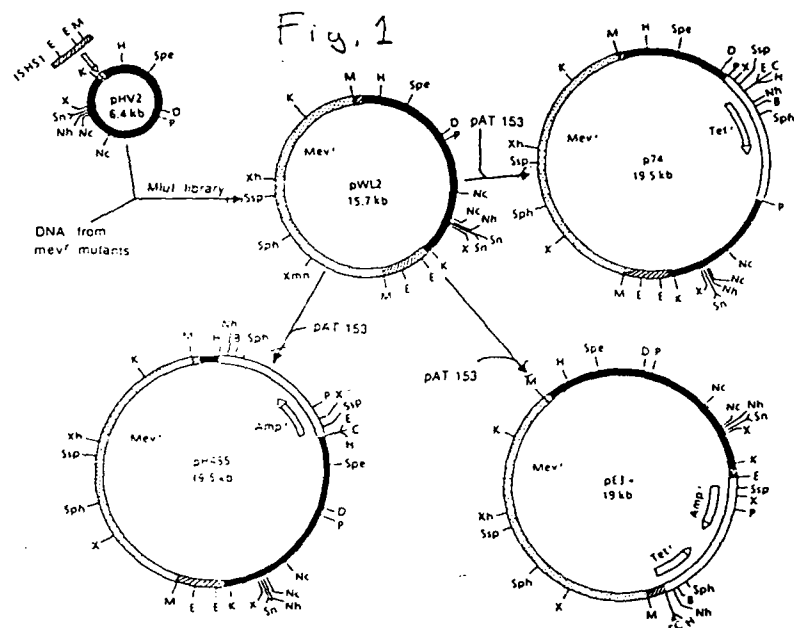
Fig. 1

Fig 2

F. g 3

Fig. 1

pHV2 6.4 kb

pWL2 15.7 kb

p74 19.5 kb

pH4SS 19.5 kb

pE3+ 19 kb

DNA from mev⁻ mutants

Mlul library

pAT 153

Fig 2

pH4SS 13.5 kb

M13-tg131 Mev 10.5 kb

pE3+ Sph I deletion

pE3+ ΔS Kpn I deletion

Kpn I / Sph I

M13 tg131

pE3+ ΔKΔS 7 kb

Bam HI / Sph I

pH4SSΔM 11.5 kb

pWL101 15 kb

pWL102 11.2 kb

deletion Bam HI / Nco I

Kpn I deletion

Fig 3

Constructions		thousands of mev⁻ transformants / ug DNA
pWL101		5
101Δ CSpe		0.2
101Δ CD		0.2
101Δ CSn		0.1
101Δ E		0.3
101Δ BM		6
101Δ K		5
101Δ BSn		6
101Δ BNc		9
101Δ BD		0.1
101Δ BSpe		0.3
101Δ H		0.3

Fig. 1. Restriction maps of pWL2 and hybrids constructed with pWL2 and pAT153. pWL2 is the mevinolin-resistance-conferring plasmid isolated from transformant M9 . Solid shading indicates pHV2 sequence, hatched shading indicates ISH51 sequence, and the dotted region is the mevinolin-resistance-conferring MluI fragment of chromosomal DNA. The unshaded region in pH455, pP74 and pE3+/pE3- is the pBR322 derivative pAT153, linearized with HindIII, PstI or EcoRI, respectively, and ligated into the corresponding sites on pWL2. Restriction endonuclease designations, in this and subsequent figures are: B, BamHI; C, ClaI; D, DraIII; E, EcoRI; H,

HindIII; K, KpnI; M, MluI; Nc, NcoI; Nh, NheI; P, PstI; Spe, SpeISph, SphI; Sn, SnaBI; Ssp, SspI; X, Xmn; Xb, XbaI; Xho, XhoI

Fig. 2. Construction of pWL101. The mevinolin-resistance fragment was excised from pH455 with MluI, to produce pH455ΔM. This was digested with BamHI and SphI, and ligated with BamHI-SphI digested M13-tg131-mev, which was obtained by ligation of the 3.5 kbp KpnI/SphI mevinolin resistance determinant of pE3+ into M13-tg131 vector from Amersham.

Fig. 3. Deletion analysis of pWL101. Deletions were made by digesting pWL101 with the indicated enzymes, religating and transforming E. coli DH5α, with selection for ampicillin resistance.

A. Schematic representation of DNA remaining after deletion. pWL101 is shown as linearized at its unique ClaI site. Mevinolin resistance region, and ampicillin-resistance region of pAT153 (between broken lines) are not displayed, as these are retained in all deletions. Shading as in Figs. 2 and 3. Frequencies of transformation of WFD11 to mevinolin resistance obtained with each deleted plasmid are indicated. **B.** Size analysis of deleted plasmids, prepared in E. coli DH5α. Plasmids were linearized with XbaI, EcoRI or HindIII, and resolved on a 0.7% agarose gel. **C.** Preparations of total DNA obtained with mevinolin resistant clones resulting from transformation with some of the deleted plasmids shown in 4B. Two independent transformants are presented for each deleted plasmid. **D.** Southern hybridization of gel shown in 2C, with pAT153 DNA as probe.